Remarks

This Amendment corrects non-substantive errors noticed in the application after the allowance.

Inaccuracies in the application have been noticed at page 22 lines 16, 17 and 25 of the application as filed ([paragraph [0090], first, second and fourth sentences, of the published application). Specifically, the words "plaques" and "lymphocytes" were misspelled and it is not correct that the cited Outram SV paper used cyclopamine to show that termination of the hedgehog/smoothened signaling was shown in the prior art to be necessary for the conversion of CD4-CD8 double negative T lymphocytes to the CD4-CD8 double positive T lymphocytes. A different specific inhibitor of hedgehog/smoothened signaling was used (a neutralizing monoclonal anti-Hh antibody). The Outram SV et al paper was filed with the IDS mailed 1/16/2004 (page 3, listing page 3 of 3, item CB), and the pages 187-189 are attached hereto for confirmation (see the paragraph bridging page 188 to 189). The specification has been corrected to change "cyclopamine" to "by treatment with an anti-Hh neutralizing monoclonal antibody" to correct this error.

A second error, page 28, line 28 of the application as filed (paragraph [0101] of the published application US 2004/0072913 A1) is an obvious typographical error. There is a erroneous substitution of the number "5" for the letter "S" in the page numbers for the King LE Jr. et al citation. The same error occurs in the listing of the document in the IDS mailed 1/16/2004, listing page 2 of 3, item CF. However the undersigned's records show that the correct document was in fact filed with the IDS. The correct page numbers are "10S-12S." That correction is made herein. A copy of the first page of King LE Jr. et al is also attached for confirmation.

The errors are correctable under Patent Office Rule 312. They are specification amendments that do not require an additional search, require only a cursory review of the record, and do not involve materially added work on the part of the Office such as checking excessive editorial changes in the specification. See MPEP 714.16. Entry is requested prior to patent issuance.

Respectfully submitted,
VIDAS, ARRETT & STEINKRAUS

Date: July 24, 2009

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Attachments

Outram SV et al (2000) *Immunity* 13: pages 187-189.
King LE Jr. et al (1990) *Journal of Investigative Dermatology* 95: page 10S

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Immunity, Vol. 13, 187–197, August, 2000, Copyright ©2000 by Cell Press

Hedgehog Signaling Regulates Differentiation from Double-Negative to Double-Positive Thymocyte

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Summary

The hedgehog (Hh) signaling pathway is involved in the development of many tissues. Here we show that sonic hedgehog (Shh) is involved in thymocyte development. Our data suggest that termination of Hh signaling is necessary for differentiation from CD4$^-$CD8$^-$ double-negative (DN) to CD4$^+$CD8$^+$ double-positive (DP) thymocyte. Shh is produced by the thymic stroma, and Patched and Smoothened (Smo), the transmembrane receptors for Shh, are expressed in DN thymocytes. A neutralizing monoclonal antibody against Shh increases differentiation of DN to DP thymocytes, and Shh protein arrests thymocyte differentiation at the CD25$^+$ DN stage, after T cell receptor β (*TCRβ*) gene rearrangement. We show that one consequence of pre-TCR signaling is downregulation of Smo, allowing DN thymocytes to proliferate and differentiate.

Introduction

The hedgehog (Hh) family of secreted proteins plays a major part in many patterning processes during animal development. Hh was first identified in *Drosophila*, where its mutations disrupt the polarity of larval segments (Nusslein-Volhard and Wieschaus, 1980). Three mammalian family members have been identified—sonic (Shh), desert (Dhh), and indian hedgehog (Ihh) (reviewed by Hammerschmidt et al., 1997). Hh proteins signal to nearby cells through two transmembrane proteins—Patched (Ptc) and Smoothened (Smo).

Ptc is thought to be the primary receptor for Hh, but expression of Smo is required for cells to transduce a hedgehog signal. In the absence of a Hh ligand, Ptc inhibits the activity of Smo, which in the absence of Ptc is constitutively active (reviewed by Ingham, 1998). The binding of the ligand relieves the inhibition (Murone et al., 1999), so that Smo can relay the signal into the target cell.

At the end of the signaling pathway are the Gli family of zinc finger transcription factors. Three *gli* genes have been described in vertebrates, *gli-1*, *-2*, and *-3*. Transcription of both *gli-1* and *gli-2* genes is upregulated upon Hh signaling (reviewed by Ruiz i Altaba, 1999). Regulation of the Hh signaling pathway is complicated (reviewed by Ingham, 1998). Truncated variants of Gli proteins exert positive or negative effects on target gene transcription (Ruiz i Altaba, 1999). *ptc* is itself a target gene of Hh signaling (Goodrich et al., 1996), providing negative feedback regulation (Goodrich et al., 1999).

The expression of bone morphogenic protein 4 (Bmp-4), a member of the TGFβ family of secreted proteins and a mammalian homolog of *Drosophila* decapentaplegic (dpp), may serve as an indicator of Hh signaling (reviewed by Hammerschmidt et al., 1997). Here we show that Shh, Ptc, Smo, Hedgehog-interacting protein (HIP), Gli-1, Gli-2, Gli-3, and Bmp-4 are expressed in the thymus and that the Hh signaling pathway regulates T cell development.

During T cell development, thymocytes pass through a series of stages, which can be defined in terms of cell surface expression of CD4 and CD8. CD4$^-$CD8$^-$ double-negative (DN) thymocytes progress to the CD4$^+$CD8$^+$ double-positive (DP) stage and then to mature CD4 or to CD8 single-positive (SP) T cells. The DN population can be further subdivided: CD44^{++}CD25$^-$ cells acquire CD25 expression, lose CD44 expression, and finally become cycling CD25$^-$CD44$^-$ DN cells before differentiating to DP cells. This sequence of developmental stages can be summarized as: CD44^{++}CD25$^-$ → CD44^{++}CD25$^+$ → CD44$^-$CD25$^+$ → CD44$^-$CD25$^-$ DN → DP → SP (reviewed by Haks et al., 1999). Thymocytes may pass through an immature CD8$^+$ SP stage before becoming DP cells. These CD8$^+$ immature single-positive (ISP) thymocytes are particularly evident during fetal development where the CD25$^-$CD44$^-$ DN population may be absent (Andjelic et al., 1993). Both the CD25$^-$CD44$^-$ DN population and the ISP population are proliferating cells that have been selected for successful T cell receptor β (*TCRβ*) rearrangement. The transition from a DN to DP cell depends on the expression of a functional pre-TCR complex (reviewed by von Boehmer et al., 1998). This study focuses on the main αβTCR T cell lineage and the transition from a DN to DP cell. At this stage, the pre-TCR signals for allelic exclusion of the *TCRβ* locus, proliferation, and differentiation (reviewed by von Boehmer et al., 1999), although the downstream transduction pathways that regulate these three outcomes of pre-TCR signaling diverge (Gaertner et al., 1999). Fetal thymus organ culture (FTOC) provides an ideal in vitro system to study thymocyte development, as embryonic day 14 (E14) fetal thymus does not contain cells that have passed beyond the CD25$^+$ DN stage, and these precursor cells can differentiate in culture through all normal stages of thymocyte development to produce mature functional T cells (reviewed by Jenkinson and Owen 1990).

Results

Molecules Involved in Hh Signaling Are Expressed in the Thymus

We assessed expression of RNAs encoding Shh, Ihh, Dhh, Ptc, Smo, HIP, (Chuang and McMahon, 1999), Gli-1,

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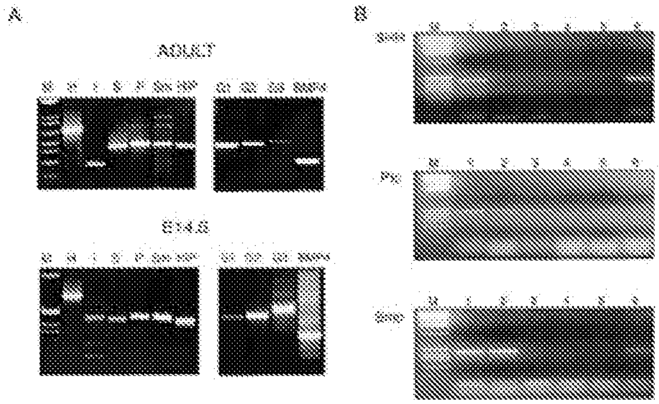

Figure 1. RT-PCR Analysis of Expression of Components of the Hedgehog Signaling Pathway in the Thymus

(A) RT-PCR analysis of expression of HPRT (H), Ihh (I), Shh (S), Ptc (P), Smo (Sm), HIP, Gli-1 (G1), Gli-2 (G2), Gli-3 (G3), and Bmp-4 in whole adult thymus and E14.5 fetal thymus.
(B) Expression of Shh, Ptc, and Smo in 1 = total thymus, 2 = CD25^{+} DN, 3 = DP, 4 = CD4SP, 5 = CD8SP, 6 = deoxyguanosine-treated thymus.

Gli-2, and Gli-3, and Bmp-4 by RT-PCR on RNA prepared from whole adult thymus and E14.5 fetal thymus (Figure 1). *shh* and *ihh* RNAs were both present (Figure 1A), but we were unable to detect *dhh* RNA (data not shown). *ptc*, *smo*, and *hip* RNAs were present (Figure 1A). As Ptc functions as a receptor for Shh and Smo is involved in Shh signal transduction, their presence suggests that the Shh signaling pathway operates in the thymus. Likewise, the presence of the transcription factors Gli-1, Gli-2, and Gli-3 and the secreted protein Bmp-4, all of which are encoded by Shh target genes (reviewed by Hammerschmidt et al., 1997; Ingham, 1998), suggests that the Hh signaling pathway is active in both the adult thymus and the embryonic thymus. Relative levels of expression of the *gli* genes observed in adult thymus and embryonic thymus were markedly different, suggesting that their contribution to thymic development differs with the age of the animal (Figure 1A).

In order to determine which cell types in the thymus express the genes involved in the Hh pathway, RT-PCR was carried out on sorted populations of thymocytes (Figure 1B). We were unable to detect *shh* RNA in any of the thymocyte populations, but *shh* was detected in primary cultures of thymic stroma, derived from deoxyguanosine-treated thymic lobes, which contain largely epithelial cells (Jenkinson and Owen, 1990). *smo* RNA was detected in the DN thymocyte population only. *ptc* RNA was detected in DN, DP, and CD8 SP cells.

To confirm the developmental expression of Hh pathway genes observed by RT-PCR, we performed in situ hybridization with radioactively labeled probes recognizing the three Hh genes as well as general Hh targets (Figure 2). On E15, only *shh*, but not *ihh* or *dhh* transcripts are detectable in a uniform pattern throughout the thymus (Figures 2A and 2B and data not shown). Likewise, the *smo* signal transducer, the *ptc-2* receptor, and the transcription factors *gli-1* and *gli-2* are expressed weakly at this stage (Figures 2C–2F). The corresponding sense probes yield no discernible signal. Interestingly, *ptc-1* expression could not be assessed by this technique due to strong nonspecific thymic labeling of the sense probe.

To investigate the pattern of expression of Smo in thymocyte populations, we carried out three-color flow cytometry using antibodies against CD4, CD8, and Smo (Figure 3A). In agreement with the RT-PCR data, most of the DN cells stained brightly with the anti-Smo antibodies. Cell surface expression of Smo was downregulated in the DP and SP populations (Figure 3A).

Analysis of DN cells showed that the CD44^{+}CD25^{-} was not stained with anti-Smo antibodies (Figure 3B). This population comprises the earliest thymic precursor cells, which are not committed to the T cell lineage (Moore and Zlotnik, 1995). Smo expression was highly upregulated in the next subset of CD44^{+}CD25^{+} cells. Both the level of expression and the proportion of cells staining positive then gradually decreased in each subsequent DN population, so that although the CD25^{+} cells stained positively, in the most mature CD25^{-} DN population staining had greatly reduced. Thus, the DN thymocytes that would seem likely to respond to Shh are the CD25^{+} population. These are the DN cells in which the *rag* genes are expressed and in which TCRβ gene segments are undergoing rearrangement (reviewed by von Boehmer et al., 1999).

When we examined Smo expression on E14 thymocytes, the majority of which are CD25^{+} DN cells, we found that about 65% stained strongly with anti-Smo antibody (Figure 3C). On E17 thymocytes, cell surface Smo expression was found on DN and CD8^{+} ISP cells, but Smo expression was downregulated in the DP population (Figure 3D).

The expression of Hh proteins was assayed by immunofluorescence on frozen sections of adult murine thymus. In agreement with the RT-PCR and in situ hybridization data, Shh was detected in epithelial cells, as demonstrated by double staining with anti-cytokeratin antibodies (Figures 3E and 3F). Shh-positive thymic epithelial cells appeared as isolated cells or small cell clusters throughout the thymic parenchyma, and the highest frequency of these cells was observed in the outer cortex (data not shown). On the contrary, no coexpression of Ihh protein and cytokeratin was found (data not shown). However, in double staining with anti-laminin antibodies, we found that Ihh expression was mainly restricted to the blood vessels located in the thymic medulla (Figures 3G and 3H). Some of the small cortical blood vessels also stained with the anti-Ihh antibodies, whereas no staining was seen in the subcapsulary area (Figures 3G and 3H).

A Neutralizing Antibody against Shh Accelerates Thymocyte Development in Thymus Explants

As Ptc and Smo were both highly expressed on E14 thymocytes, we tested the effects of inhibition of the Hh pathway by culturing thymocytes with the anti-Hh

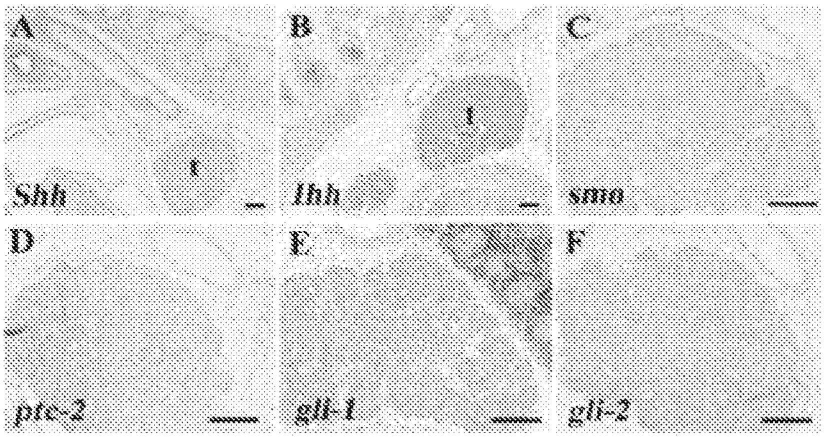

Figure 2. In Situ Hybridization Analysis of Hh and Hh Target Gene Expression in the Developing Mouse Thymus

Expression of the indicated genes was investigated in parasagittal paraffin sections of mouse embryos on E15. Bright field and corresponding dark-field images were superimposed.

(A and B) Shh, but not Ihh is expressed in the early mouse thymus (t). Note the absence of Ihh signal in the thymus in (B). The same section displays strong expression of the gene in the cartilage of the developing ribs.

(C–F) Transcripts of Hh target genes are detectable throughout the early mouse thymus. Scale bars denote 100 μm.

neutralizing mAb 5E1 (Ericson et al., 1996). E14 thymus lobes were cultured in the presence of 5 μg/ml of the purified antibody. After 3 days in culture, we found a large increase in the production of DP thymocytes in the antibody-treated cultures compared to control cultures. In the presence of the antibody, 58% of cells progressed to the DP stage, whereas only about 9% of thymocytes did so in the control cultures, and the majority of the DN cells in the control cultures still expressed CD25 (Figure 4A). Thus, neutralization of Shh greatly increased thymocyte differentiation over this 3 day time period.

In addition, in all experiments, there was a 2- to 3.5-fold increase in the number of thymocytes recovered from the cultures containing the neutralizing antibody (Figure 4C), so that, in absolute terms, between 10 and 20 times more DP cells were produced in the antibody-treated cultures than in control cultures.

As the neutralizing antibody increased the production of DP thymocytes, it seemed possible that DN thymocytes were being induced to differentiate before they had rearranged a *TCRβ* chain gene. We therefore examined the expression of the αβTCR on the DP thymocytes from the control and anti-Shh-treated cultures. Levels of expression of the TCRβ chain were very similar in both culture conditions, suggesting that the extra DP thymocytes produced in the presence of the anti-Shh antibody had rearranged their *TCRβ* gene (Figure 4B). In addition, we looked at expression of CD2 and CD24 (HSA) on the DP cells and found no differences (Figure 4B).

One explanation for the increase in DP cells in antibody-treated cultures is that neutralization of Shh stimulated proliferation of DP cells, rather than increasing the differentiation of DN cells. To test this, we added BrdU to 3 day cultures of thymus overnight and assessed BrdU incorporation into CD8^{+} cells by flow cytometry using antibodies against BrdU and CD8. In the control cultures, 40% of CD8^{+} cells (made up mostly of DP cells; see Figure 4A) had incorporated BrdU, whereas only 22% of CD8^{+} cells did so in the cultures treated with anti-Shh antibody (Figure 4D). Thus, the increase in DP cells could not be accounted for by a simple increase in their rate of proliferation, but may be due to an increase in differentiation from DN to DP thymocytes. The finding that the antibody-treated cultures contained a smaller proportion of cycling DP cells than the control cultures supports the idea that the antibody treatment had accelerated differentiation and that the DP cells, after undergoing an initial phase of proliferation, had progressed to the more mature small nonproliferating stage. In absolute terms, however, there were more BrdU^{+} cells in the antibody-treated cultures than in control cultures.

Shh Protein Arrests Thymocyte Development at the DN Stage

Given that the anti-Shh antibody increased the differentiation of DN to DP thymocytes, we tested the effect of Shh protein on thymocyte development. Human recombinant Shh modified for high activity was added to the cultures at concentrations ranging from 0.1 μg/ml to 1.8 μg/ml. After 2 days, the culture medium was changed and fresh Shh was added. After 3 days, about 35% of thymocytes in control cultures expressed CD4 and CD8, whereas in the presence of Shh the production of DP thymocytes was inhibited in a dose-dependent manner, with maximum inhibition at 0.9 μg/ml (Figure 4E). Thus, Shh arrested thymocyte development at the DN stage. These concentrations of Shh are within the predicted physiological range, as this modified Shh protein has maximum activity in a cell-based reporter gene assay at 0.3 μg/ml and is thought to be about 20–30 times more active than the unmodified Shh protein (J. Porter, personal communication). Analysis of the composition of the DN cells showed an increase in the proportion of CD25^{+}CD44^{low} DN cells and a concomitant decrease in

Epidermal Growth Factor/Transforming Growth Factor Alpha Receptors and Psoriasis

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The abnormal growth and differentiation in psoriasis is reflected in the abnormal regulation of Epidermal Growth Factor/Transforming Growth Factor Alpha (EGF/TGFα) receptor metabolism. In psoriasis and other hyperproliferative skin conditions these receptors are persistently expressed throughout the interfollicular epidermis as long as the growth stimulatory signal persists. One of the first biochemical signs of effective therapy of psoriasis is the return of the EGF/TGFα receptor pattern toward the primarily basilar distribution seen in normal human adult skin. Whether the abnormal expression of TGFα in the involved skin induces the persistent expression of EGF receptors is not known nor is the signal that causes the increased production of TGFα. Studies to determine what factors regulate EGF receptor expression and TGFα induction may yield important new insights into the pathogenesis and therapy of psoriasis. *J Invest Dermatol 95:10S–12S, 1990*

One approach to studying growth regulatory mechanisms in psoriasis is to use well-studied proteins capable of regulating normal human keratinocyte proliferation and differentiation, i.e., EGF, TGFα, and their common, but specific membrane receptor [1–3]. EGF is a small (53 amino acid) protein that directly stimulates growth and differentiation in a number of epithelial tissues, stimulates the growth of mesenchymal and endothelial cells, and has multi-functional effects such as ion secretion, muscle contraction, and cell movement [1–3]. For example, in vivo EGF/urogastrone stimulates growth of lung, corneal, tracheal, and GI tract epithelium and inhibits gastric acid secretion [3]. The primary sequence of EGF, urogastrone, TGFα, and structurally related proteins (Fig 1) has been determined, and many of them have been cloned [2]. EGF and TGFα are present in human platelets, and EGF is found in all body fluids. TGFα has overall but limited homology to EGF and is antigenically related, binds to the EGF receptor, and in some systems has different or more profound effects than EGF [3,4]. Because these and other less well-defined proteins, such as vaccinia virus growth factor (VVGF), are structurally related to EGF and bind to the EGF/TGFα receptor, this receptor may be involved in a number of seemingly unrelated diseases [2].

All known effects of EGF and TGFα are mediated by their specific receptor, and if that receptor is mutated as in the v-erb B oncogene it causes cancer or abnormal proliferation such as with psoriasis [2]. Therefore, errors in EGF/TGFα receptor metabolism such as point mutations and internalization, degradation, phosphorylation, dephosphorylation, or glycosylation defects may cause human skin diseases. Abnormalities in the EGF/TGFα receptor can occur at a number of sites, because it is a single-chain glycoprotein of approximately 170,000 kDa containing an extracellular EGF binding site, a small transmembrane portion, and a cytoplasmic intrinsic EGF stimulated tyrosine kinase, along with its phosphate acceptor sites. When EGF or related proteins bind to the extracellular portion of the EGF/TGFα receptor, the receptor autophosphorylates itself or other biologically active proteins on specific tyrosine residues to transduce extracellular signals regulating cell proliferation/differentiation. If growth factor binding is increased or decreased, or this signal is altered by known (PDGF, TGFβ) or unknown factors, then the epidermis may have abnormal growth and/or differentiation.

EGF/TGFα Receptor Localization and Metabolism The EGF/TGFα receptor is almost ubiquitous in non-hematopoietic cells. These localization data based on the binding of radioactive ligands and anti-receptor antibodies correspond well to the localization of mRNA for EGF, TGFα, and their receptor [5]. Immunoreactive and ^{125}I-EGF binding receptors were found in cell membranes of dividing and non-dividing epithelial and mesenchymal cells [6]. The receptors are expressed in a developmentally regulated manner in embryonic human skin and are differentially expressed in hair follicle formation.* Even in embryonic and neonatal skin, receptor expression appears to be under regulatory control that can be modulated and/or reversed.* In rapidly growing newborn foreskin epidermis the EGF/TGFα receptors appear to be expressed in all layers of the epidermis.* At a later time the more adult pattern with receptors primarily confined to the basal cell layers is seen. When the adult pattern is seen in the newborn period, as it is in restrictive dermopathy, this distribution is abnormal. Therefore, it is important to know that the normal patterns of receptor expression are important for both the site and the developmental age. In normal adult human epidermis EGF/TGFα receptors, as detected by binding of ^{125}I-EGF, are found in the highest concentrations on basal keratinocytes and decrease in number as keratinocytes differentiate to become the normal stratum corneum [6]. This ^{125}I-EGF binding pattern in human epidermis has been confirmed, but there may be slight variations in the pattern of immunoreactive EGF/TGFα re-

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Abbreviations:

- EGF/TGFα: epidermal growth factor/transforming growth factor alpha
- PKC: protein kinase c

* Nanney et al: J Invest Dermatol (submitted).

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